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Natural course of subjects with elevated liver tests and normal liver histology

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Abstract: BACKGROUND AIMS: Liver biopsy (LB) is performed if non-invasive work-up of liver disease is inconclusive. The examination of liver tissue occasionally reveals normal histology. Long-term follow-up of such patients has not been performed. METHODS: We identified a total 70 subjects from our LB database with elevated liver tests and normal liver histology after a mean of 90.5 ± 52.3 (range 15-216) months and conducted reassessment of medical history, physical examination, laboratory testing, ultrasound, transient elastography and LB if indicated. RESULTS: At follow-up examination, 15 (7 females (f)/8 males (m); 21.4%) subjects had normal liver tests and no further evidence of liver disease. A subset of 37 (29 f/8 m; 52.9%) subjects had persistently elevated liver tests without evidence indicating progressive liver disease but the cause thereof remained unexplained also at the follow-up visit. Three (0 f/3 m; 4.3%) subjects had consumed excessive alcohol with indicators of alcoholic liver disease. Eleven subjects (4 f/7 m; 15.7%) had developed steatosis on ultrasound examination along with weight gain and/or biochemical features of the metabolic syndrome. In addition, three (2 f/1 m) patients developed autoimmune hepatitis, one female presented with primary biliary cirrhosis. One male was diagnosed with cholangiocellular carcinoma 3 months after the initial evaluation. CONCLUSION: The clinical course of most patients was benign, but in approximately 20% of the subjects a liver disease developed. Particular attention should be given to autoimmune liver diseases in subjects with positive autoantibodies. In addition, lifestyle factors such as weight gain and alcohol consumption were associated with the manifestation of liver diseases.

DOI: https://doi.org/10.1111/liv.12935

Posted at the Zurich Open Repository and Archive, University of Zurich
ZORA URL: https://doi.org/10.5167/uzh-119318
Journal Article
Accepted Version

Originally published at:
Strasser, Michael; Stadlmayr, Andreas; Haufe, Heike; Stickel, Felix; Ferenci, Peter; Patsch, Wolfgang; Feldman, Alexandra; Weghuber, Daniel; Datz, Christian; Aigner, Elmar (2016). Natural course of subjects with elevated liver tests and normal liver histology. Liver International, 36(1):119-125.
DOI: https://doi.org/10.1111/liv.12935

Follow-up of subjects with normal liver histology identifies a substantial risk of subsequent liver diseases, particularly nonalcoholic fatty liver

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Disclosures: None of the authors have any potential conflicts of interest with regard to this study.

Key words: liver biopsy; NAFLD; normal histology; BMI, liver;

Acknowledgement: Elmar Aigner is supported by PMU-Forschungsförderungsfonds (E-13/17/086-AIG). Support from Spar Austria to CD is gratefully acknowledged.

Abbreviations: eLE – elevated liver enzymes; NAFLD – nonalcoholic fatty liver disease;

Abstract:

Background: Liver biopsy (LB) is performed to determine the etiology of elevated liver enzymes (eLE) if non-invasive work-up has remained inconclusive. The pathological examination of liver tissue not infrequently reveals normal histology. To date, no systematic long term follow-up of such subjects has been performed.

Methods: We identified a total of 87 subjects from our LB database with eLE and normal liver histology. Of these, 70 (84.2%, 39 women, 18-72yrs; 31 men, 18-74yrs) were available for a detailed follow-up evaluation after 90.5±52.3 (range 15-216) months including medical history, physical examination, extensive laboratory testing, ultrasound, transient elastography and LB if indicated.

Results: At the follow-up examination, 15 (7/8; 21.4%) subjects had normal LE and did not have any evidence of liver disease. Thirty-seven (24/6; 52.9%) subjects had persistently eLE but the cause remained enigmatic also at the follow-up visit without evidence indicating progressive liver disease. Three (0/3; 4.3%) subjects had consumed excessive amounts of alcohol with indicators of alcoholic liver disease. Eleven subjects (4/7; 15.7%) subjects had developed steatosis on ultrasound examination along with weight gain and biochemical features of the metabolic syndrome. Additionally, 2 female subjects developed autoimmune hepatitis, one female primary biliary cirrhosis, and one male subject was diagnosed with cholangiocellular carcinoma.

Conclusion: In approximately 1 in 5 subjects, eLE may be related to a liver disease that is missed on LB. Particular attention should be paid to the onset of autoimmune diseases in women. Additionally, eLE indicative may herald consecutive hepatic steatosis if accompanied by weight gain and laboratory components of the metabolic syndrome. Although discharge from care is appropriate for most subjects, adopting a routine of follow-up for patients with eLE and normal histology appears clinically advisable.

Introduction: Microscopic examination of a liver biopsy specimen as a means to identify causes and mechanisms of liver injury was introduced by Hans Popper in the first half of the 20th century and paved the way to modern hepatology.[1] Although improved serological testing and non-invasive assessment of fibrosis have significantly decreased the clinical need to perform liver biopsies for previous standard indications in recent years, liver biopsy is still performed if the etiology of elevated liver enzymes (eLE; i.e. transaminases – aspartate aminotransferase, AST and alanine aminotransferase, ALT or cholestasis indicating enzymes – alkaline phosphatase, AP and gamma glutamyl-transpeptidase, GGT) cannot be determined by non-invasive means.

Percutaneous liver biopsy is still the gold standard for establishing a diagnosis and the staging of liver diseases although its limitations such as sampling error or inter-observer variability are well-recognized.[2] On clinical grounds, the most frequent causes expected and identified are seronegative autoimmune diseases (autoimmune hepatitis - AIH, primary biliary cirrhosis – PBC, primary sclerosing cholangitis - PSC, drug-induced liver injury – DILI, nonalcoholic steatohepatitis – NASH, secondary biliary cirrhosis, secondary sclerosing cholangitis, alcohol-related liver diseases or amyloidosis.[3] Of note, a small proportion of liver biopsies obtained for these diagnostic indications do not reveal any abnormalities of the liver tissue and are assessed as normal liver parenchyma by the pathologist. The prevalence of normal liver biopsies in case of unexplained elevations of liver enzymes ranged between approximately 3.3%,[4] 6.0%[3] and 10%.[5]

To our knowledge, no systematic investigation of the subjects with normal histology at index liver biopsy has been performed to determine the long term clinical outcome of these patients. It is the common clinical routine to discharge these patients from medical care with the assurance that the liver is to be regarded as healthy and further investigations are not required. We therefore aimed to identify and follow-up all patients at two participating centers

who had received the diagnosis of a “normal liver” after liver biopsy in order to generate data of the long term clinical outcome of these patients.

Material and Methods

Subjects: Between January 1996 and December 2013 approximately 1400 diagnostic liver biopsies had been performed at two centers for various indications. We aimed to identify subjects who had percutaneous liver biopsies performed due to repeated elevations of liver enzymes after exclusion of history of relevant alcohol intake (>20g/day), viral hepatitis, autoimmune hepatitis, primary biliary cirrhosis, Wilson disease, alpha-1-antitrypsin deficiency, hereditary hemochromatosis (HFE-associated) or known steatogenic medication. Ultrasound examination at the time of LB had revealed normal liver echogenity and parenchymal structure in all subjects. Additionally, these patients were not allowed to have signs of heart or renal insufficiency or suffer from cancer, auto-immune diseases or systemic infections. A detailed chart review identified 87 subjects with normal liver histology who met the above mentioned criteria. Of these, 6 subjects who met the criteria but in retrospect did not have representative liver biopsy specimens were not contacted. A representative index LB was defined as length of specimen ≥15mm or 8-10 portal tracts.[6] Thus, 81 were contacted and invited for a follow-up visit and 70 of these were re-evaluated as detailed below.

Right upper quadrant US examination was performed using an ATL HDI 5000 machine (Phillips Medical Systems, Vienna, Austria). The examinations were carried out by one of five physicians with 5–25 years of experience. The liver was considered ‘normal’ if the echogenicity was homogenous and similar to or slightly higher than the echogenicity of the renal parenchyma with a normal vascular and biliary architecture. The liver was considered ‘fatty liver’ if areas of significant increased echogenicity in relation to the renal parenchyma were found. The severity of sonographic steatosis was not graded. Transient elastography was

performed using Fibroscan® (Echosens™, Paris, France) according to the company instructions.

Written informed consent was obtained from all study participants and the study was approved of by the local ethics committee (Ethikkommission des Landes Salzburg) and was conducted in accordance to the ethical standards of the Helsinki Declaration of 1975 (revised in 1983).

Laboratory Evaluation: Examinations were performed at the time of the liver biopsy (January 1996 to Dezember 2012) and at the follow-up visit (January 2014 to June 2014). Venous blood was drawn following an overnight fast for determination of liver function tests, a full blood count, serum iron status including ferritin, transferrin, transferrin saturation and serum iron, C-reactive protein, erythrocyte sedimentation rate (ESR), fasting glucose and lipids hepatitis B and C serology and PCR, autoantibody screening (ANA, ASMA, LC, LKM, AMA), by standardized automated laboratory methods.

Histologic examination of liver biopsy samples: All liver biopsy specimens were routinely fixed in buffered formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin, Mallory trichrome stain for morphological evaluation and Perls´ stain was used for determination of liver iron load. All liver biopsies were routinely analysed independently by two pathologists and judged as normal liver tissue, thus no inflammatory infiltrate was present, no steatosis, no fibrosis and negative iron stain were recorded as detailed previously. Hepatic iron and copper content were determined by automated mass spectroscopy and calculated as µg/g dry weight in 32/65 patients as clinically indicated and were also within limits of normal.[7]

Statistical analysis: Statistical analyses were carried out using SPSS (SPSS 13.0) statistics package. Calculations for differences between the various groups were carried out by Student's t-test or by non-parametric Kruskal-Wallis test in case of non-Gaussian distribution of parameters. Associations among the parameters in the different groups were calculated

using Spearman's rank correlation technique. Changes of various parameters over time in respective groups were analyzed using paired t-tests.

Results

Patients' baseline characteristics: The patients' baseline characteristics are summarized in Table 1. They were without immunologic, infectious, malignant or metabolic diseases and thus generally healthy apart from persistently eLE. The median time to follow-up was 90.9±50.4 months (range 13 – 208 months).

	female	male
No	39	31
Age (yrs)	46.1±15.1	39.2±12.5
BMI (kg/m²)	24.2±2.6	24.7±4.0
AST (IU/L)	44.6±29.8	51.6±28.1
ALT (IU/L)	69.9±45.2	86.5±56.4
AP (IU/L)	143.6±100.9	127.4±63.0
GGT (IU/L)	222.5±130.2	164.0±63.4
Cholesterol (mg/dL)	226.3±46.1	229.5±48.3
Triglyceride (mg/dL)	109.1±46.3	119.1±54.10
Ferritin (ng/mL)	157.3±121.8	139.2±110.4
Fasting glucose (mg/dL)	87.4±13.2	82.2±11.5
Thrombocytes (G/L)	243.6±69.2	234.9±62.6

Table 1: Baseline characteristics of the study cohort. *Abbreviations: BMI – body mass index; AST – aspartate aminotransferase; ALT – alanine aminotransferase; AP – alkaline phosphatase; GGT – gamma glutamyltranspeptidase;*

Clinical results at follow-up visit: All patients were assessed by using current standard laboratory, clinical and ultrasound equipment, transient elastography (Fibroscan) in order to non-invasively assess the presence and stage of potential liver disease.

At the follow-up examination, 15 (7/8; 21.4%) subjects had normal LE and did not have any evidence of liver disease. Thirty-seven (24/6; 52.9%) subjects had persistently eLE but the cause remained enigmatic also at the follow-up visit without evidence indicating progressive liver disease. Three (0/3; 4.3%) subjects had consumed excessive amounts of alcohol with indicators of alcoholic liver disease. Eleven subjects (4/7; 15.7%) subjects had

developed steatosis on ultrasound examination along with weight gain and biochemical features of the metabolic syndrome. Clinical and biochemical changes in these subjects comprised an increase in BMI, serum triglycerides, ALT, GGT, and ferritin along with a decrease in serum HDL concentrations. These changes were significantly different from those observed in subjects who were had normalized or were unchanged with regard to liver disease biochemically and clinically. These changes are reported in detail in Table 2. Additionally, 2 female subjects developed autoimmune hepatitis, one female primary biliary cirrhosis, and one male subject was diagnosed with cholangiocellular carcinoma. One male subject with normal LE and elastography had acquired detectable anti-HBc and anti-HBs with undetectable HBs-Ag and HBV-DNA consistent with HBV infection and spontaneous seroconversion during the follow-up period. As this patient had normal LE and normal elastography he was allocated to the appropriate group for calculations.

Transient elastography: Liver elastography measurement using Fibroscan yielded a liver elasticity of 4.5±0.7 kPa in normalized subjects, 4.3±0.6 kPa in unchanged subjects and 6.0±2.3 kPa in NAFLD subjects. Thus, elasticity was significantly increased in NAFLD subjects compared to the other two groups (P<0.001; ANOVA). It was 14.8 kPa (PBC) and 4.1kPa (AIH) in subjects who were diagnosed with autoimmune disease. Among subjects with alcoholic liver disease, 7.1 kPa and 4.7 kPa were measured. Fibroscan results could not be obtained from 2 subjects, one with PBC and one with alcoholic liver disease and BMI 36.1 kg/m².

	normalized	**unchanged**	**NAFLD**	**P (ANOVA)**
	15	37	11	
BMI BL	24.0±2.9	24.1±3.4	26.1±3.3	
BMI FU	23.7±2.5	24.8±3.4	29.0±4.5	
dBMI	-0.32±1.16	0.66±1.50	2.83±2.25	<0.001
ALT BL	56.7±54.1	66.2±27.5	98.8±51.8	
ALT FU	23.2±9.2	55.2±32.0	85.0±53.2	
dALT	-33.5±50.4	10.9±27.9	13.7±46	<0.001

AST BL	33.7±24.1	44.1±19.5	65.5±37.8	
AST FU	24.4±6.8	39.9±18.7	60.8±16.7	
dAST	-9.3±13.5	-4.6±0.9	-4.6±21.1	0.427
GGT BL	133.0±134.7	183.3±102.8	233.4±136.7	
GGT FU	51.3±38.1	209.7±108.7	279.5±109.8	
dGGT	-81.5±107.1	26.4±95.2	46.1±155.2	<0.001
TG BL	88.3±37.5	119.4±50.0	140.9±59.3	
TG FU	99.7±42.3	126.9±59.9	169.4±68.4	
dTG	11.4±4.4	7.5±16.0	28.5±9.1	0.027
HDL BL	65.1±15.8	67.2±18.7	67.5±18.4	
HDL FU	66.6±20.0	66.1±19.6	59.1±14.8	
dHDL	1.5±4.2	-1.1±1.0	-8.4±3.6	<0.001
Ferritin BL	153.5±110.0	151.8±104.2	153.8±127.2	
Ferritin FU	109.2±45.5	152.2±133.2	183.0±150.2	
dFerritin	-44.3±14.2	0.36±28.9	29.2±22.9	<0.001

Table 2: Antropomorphic and biochemical differences between subjects that had normal liver, unchanged elevation of liver enzymes and those who developed nonalcoholic fatty liver. *Abbreviations: BL – baseline; FU – follow-up; BMI – body mass index; AST – aspartate aminotransferase; ALT – alanine aminotransferase; AP – alkaline phosphatase; GGT – gamma glutamyltranspeptidase;*

Baseline predictors of the subsequent clinical course: In order to identify clinical or biochemical parameters predictive of the further clinical development multivariate regression analysis was performed after adjustment for sex and time to follow-up visit. Patients were grouped as 0 – normalized, 1 – unchanged, 2 – NAFLD at follow-up visit. Parameters with significant associations in the univariate analysis entered the multivariate model. Thus, associations with BMI, ALT, GGT and triglyceride serum concentrations remained intact in the multivariate model. Of these, baseline higher ALT and AST values were predictive of new-onset NAFLD. Lower serum triglyceride and total cholesterol concentrations were independently associated with normalization of transaminases at follow-up.

Discussion: Liver biopsy is still the – although imperfect – gold standard for the assessment of liver diseases. It allows for grading and staging of a disease and frequently elucidates the underlying etiology when extensive laboratory tests retrieve indefinite results. Although normal liver histology is not infrequently obtained in subjects who undergo biopsy for elevated liver enzymes of unknown origin, to our knowledge the long term outcome of such patients has not yet been assessed. As these patients are commonly discharged from further hepatology specialist care with the assurance of absence of a relevant liver disease, we aimed to systematically assess the clinical outcome of these patients. Our data demonstrate that despite normal liver histology at the time of the indicated index biopsy, important entities such as autoimmune diseases (AIH, PBC), cancer (undiagnosed cholangiocellular carcinoma) and most frequently non-alcoholic fatty liver disease became clinically evident in approximately one fifth of the subjects. Clinical judgement suggests that eLE in these subjects were likely related to these entities already at baseline. As the rate of normal liver biopsies in our study is comparable to rates reported from other studies, our results may be applicable to the general clinical setting in Western countries.

Approximately 80% of all study subjects either had normal LE and no clinical evidence of disease or still had eLE but also no evidence of progressive underlying liver disease. Hence, the largest proportion of patients with normal LB did not display clinically relevant evidence of liver disease several years after the initial biopsy. However, several distinct longitudinal clinically relevant courses can be derived from the observations of our study population. And treating clinicians should be aware in the daily routine that these may affect approximately 20% of subjects.

These distinct courses are as follows: 1) Autoimmune liver diseases in women: Two women who were biopsied due to mildly elevated transaminases and low-titer autoantibodies were finally diagnosed with AIH 12 and 26 months after the index biopsy. One of these then

was already at a progressed stage of AIH. Similarly, one woman who had undergone biopsy for unexplained elevation of AP and GGT and negative AMA received the diagnosis of PBC at the time of recall. We therefore suggest that particularly in female subjects with a suspicion of autoimmune liver disease, a normal biopsy may not definitely exclude these diseases. They should likely be followed despite a normal liver biopsy at an earlier time point as the full clinical and biochemical picture of these diseases may follow the elevation of liver enzymes.

2) NAFLD in association with further weight gain: NAFLD has become the most frequent liver in recent decades due to the epidemic rise in obesity and it is not surprising that subjects receiving the diagnosis NAFLD were the largest group among those with definite diagnosis at follow-up. We consider some observations with regard to the disease spectrum of NAFLD worth mentioning and clinically relevant. In retrospect, 11 of 11 patients had laboratory and clinical signs suggestive of NAFLD already at the time of the index biopsy. However, liver tissue revealed no steatosis or other features potentially linked to NAFLD such as ballooning, inflammation, fibrosis or iron deposition. At follow-up, they had gained further weight and developed unequivocal steatosis on US examination and other characteristics of the metabolic syndrome. In these cases, NAFLD was clinically diagnosed. On the other hand, 5 patients whose clinical constellation would likewise have suggested NAFLD effectively changed lifestyle and lost weight compared to the time of the biopsy. In these subjects, normal US, elastography and liver enzymes were observed at follow-up. Taking these 2 groups together, we suggest that weight and lifestyle factors were likely responsible for the elevation of liver enzymes at baseline, even in the histological absence of steatosis. One is tempted to speculate that these subjects represent a novel segment in the disease spectrum of NAFLD. In NAFLD, a large proportion of patients with significant steatosis and even NASH present with normal transaminases. Our observations indicate that on the other end of the spectrum, eLE together with risk indicators suggestive of NAFLD may be clinically apparent prior to hepatocellular steatosis. In this group, effective lifestyle

modifications and weight loss was associated with the normalization of previously eLE. Conversely, further weight gain in the absence of effective lifestyle modification may aggravate the clinical picture with hepatic steatosis to become apparent subsequently. In NAFLD pathogenesis, the two-hit hypothesis with steatosis as the necessary first hit,[8] has been replaced by the multiple-parallel-hit hypothesis.[9] One of the implications of the multiple-parallel-hit hypothesis is the fact that NAFLD may in fact comprise several similar yet distinct clinical manifestations as has also been suggested by analysis of fibrosis patterns in NAFLD.[10] Our observations strongly support the multiple-parallel-hit hypothesis, as elevation of liver enzymes indicating NAFLD were present even in the absence of hepatocellular steatosis.

In addition to these three large groups, one young male who received a liver biopsy due to unexplained elevation of AP and GGT was diagnosed with metastatic cholangiocellular carcinoma only three months later and died few weeks after the diagnosis. No evidence thereof had been obtained from CT, MRI, MRCP and ERCP immediately prior to the biopsy. Even in retrospective analysis of imaging modalities there was no diagnostic hint that would have changed the clinical course.

A change of drinking habits with significantly higher alcohol intake compared to the time of biopsy was estimated to be the cause for deterioration in liver enzymes and development of steatosis in 3 subjects (1 female, 2 male). Furthermore, one male subject had acquired and cleared contact with hepatitis B virus accounting for one hepatitis B infection in the whole cohort representing approximately 522 patient years.

It appears noteworthy, that in this relatively large cohort we were not able to identify a single individual with progressive liver disease in whom the cause had remained enigmatic. This finding may represent an important aspect to the entity of cryptogenic liver disease since these data suggest that truly cryptogenic liver disease may be a very rare diagnosis at least if a normal biopsy has been obtained. It has been well documented that genetic modifiers impact

on liver enzymes. (Chambers wass, Natgen 2012, und yuan waterworth Hum mol gen 2008) Even at follow-up, subjects with eLE and no clinical evidence of liver disease comprised the largest group in our analysis. One may assume that underlying genetic variations contribute to the elevation of LE particularly in this group, since potential explanations for normalization or deterioration could be identified in the remaining subjects.

In summary, our observations suggest that a normal liver biopsy performed for diagnostic purposes in case of unexplained elevation of liver enzymes does not definitely exclude an underlying yet only subsequently manifesting liver diseases. In particular, attention should be paid to the onset of autoimmune diseases in women. Additionally, eLE indicative of NAFLD may herald consecutive steatosis and weight gain. These data suggest that elevation of transaminases without histological steatosis linked to metabolic risk factors may herald the future onset of NAFLD or even represent a unique entity in the spectrum of NAFLD. Although discharge from care is clinically appropriate for most subjects, adopting a routine of follow-up for patients with eLE and normal histology appears advisable.

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